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Expression of neuropeptide- and hormone-encoding genes in the *Ciona intestinalis* larval brain

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ABSTRACT

Despite containing only approximately 330 cells, the central nervous system (CNS) of *Ciona intestinalis* larvae has an architecture that is similar to the vertebrate CNS. Although only vertebrates have a distinct hypothalamus—the source of numerous neurohormone peptides that play pivotal roles in the development, function, and maintenance of various neuronal and endocrine systems, it is suggested that the *Ciona* brain contains a region that corresponds to the vertebrate hypothalamus. To identify genes expressed in the brain, we isolated brain vesicles using transgenic embryos carrying *Ci-β-tubulin(promoter)::Kaede*, which resulted in robust Kaede expression in the larval CNS. The associated transcriptome was investigated using microarray analysis. We identified 565 genes that were preferentially expressed in the larval brain. Among these genes, 11 encoded neurohormone peptides including such hypothalamic peptides as gonadotropin-releasing hormone and oxytocin/vasopressin. Six of the identified peptide genes had not been previously described. We also found that genes encoding receptors for some of the peptides were expressed in the brain. Interestingly, whole-mount *in situ* hybridization showed that most of the peptide genes were expressed in the ventral brain. This catalog of the genes expressed in the larval brain should help elucidate the evolution, development, and functioning of the chordate brain.

Introduction

Among urochordates (tunicates)—the closest living relatives of vertebrates—*Ciona intestinalis* is increasingly being used as a model organism in the field of developmental neurobiology (Meinertzhagen and Okamura, 2001; Wada and Satoh, 2001; Meinertzhagen et al., 2004). The fertilized *Ciona intestinalis* egg develops into a tadpole larva with a simplified chordate body plan, including a dorsal neural tube in the trunk and a notochord in the tail (Satoh, 2003; Satoh et al., 2003). The neural tube forms as the left and right edges of the neural plate curl together, a developmental process that is evolutionarily conserved in urochordates and vertebrates (Satoh, 1994).

Ciona intestinalis is the seventh published animal genome; the ~120-Mbp euchromatin region is estimated to contain ~16,000 protein-coding genes (Dehal et al., 2002; Satou et al., 2008). In addition, analyses of more than one million ESTs have provided the foundation for gene models and associated transcriptomes (Satoh et al., 2003). More recent studies have begun to elucidate the complex gene regulatory networks underlying the formation of the *Ciona* larval central nervous system (CNS) (Moret et al., 2005; Ikuta and Saiga, 2007; Imai et al., 2009).

The sea squirt has been used by developmental neurobiologists because of its small cell number and extensively characterized CNS (Takamura, 1998; Imai and Meinertzhagen, 2007). The CNS of an ascidian larva is subdivided morphologically along the anterior–posterior axis into the sensory vesicle, which contains the otolith and ocellus, the neck region, the visceral ganglion with five pairs of motoneurons, and the caudal nerve cord comprising primarily ependymal cells (Meinertzhagen et al., 2004). The sensory vesicle, neck region, visceral ganglion, and nerve cord are composed of approximately 215, 6, 45, and 65 cells, respectively. Thus, these tissues contain approximately 330 cells, less than 100 of which likely are neurons (Nicol and Meinertzhagen, 1991; Imai and Meinertzhagen, 2007). After fertilization, a larva develops from no more than 13 divisions (Cole and Meinertzhagen, 2004). This simple nervous system provides an opportunity to study developmental neurobiology and neuroethology in chordates at the single-cell level using neuron-labeling strategies that have been widely employed in invertebrate systems (Meinertzhagen et al., 2004).

Transcriptional factor gene expression in the CNS also supports the presence of conserved brain structures between ascidians and other

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animals. Early gene expression studies revealed that *Otx*, *Pax2/5/8*, and *Hox* are expressed in ascidian larvae with a tripartite organization along the neuroaxis (Wada et al., 1998). These results were followed by studies showing the expression of *Pax2/5/8*, *engrailed*, and *FGF8/17/18*, *Dmbx*, *Lhx3* and *Phox2* between the areas positive for *Otx* and *Hox* genes, an organization that is similar to expression profiles observed in the vertebrate isthmus (Imai et al., 2002; Jiang and Smith, 2002; Dufour et al., 2006; Ikuta and Saiga, 2007). Moret et al. (2005) examined the expression patterns of genes that are homologous to vertebrate ventral forebrain markers; the authors found that *Ci-Otp*, *Ci-Meis*, *Ci-Hif*, *Ci-Nkx2.1*, *Ci-Six3/6*, *Ci-FoxB*, and *Ci-FoxHa* were expressed in the ventral sensory vesicle. *Otp*, *Meis*, and *Nkx* are specifically expressed in the vertebrate hypothalamus, the most ventral part of the diencephalon. Therefore, it appears that the ventral region of the *Ciona* brain corresponds to the vertebrate hypothalamus, although further functional data is needed.

The hypothalamus—the center of the vertebrate endocrine system—synthesizes and secretes such neurohormones as gonadotropin-releasing hormone (GnRH) and oxytocin/vasopressin via the pituitary gland. These short peptides, which are produced via proteolytic cleavage of pre-prohormone precursors, are primarily ligands for G-protein-coupled receptors (GPCRs). Neuropeptides and peptide hormones contribute to a number of physiologic systems, including stress responses, reproduction, and homeostasis. Peptide hormones also regulate growth and differentiation during embryogenesis (Sanders and Harvey, 2008).

The close phylogenic relationship between ascidians and vertebrates has led researchers to identify and characterize ascidian neuropeptides and hormone peptides, including GnRH, tachykinins, oxytocin/vasopressin family peptides, calcitonin/calcitonin gene-related peptides, and insulin and other related peptides (reviewed by Campbell et al., 2004; Satake et al., 2003; Sherwood et al., 2006; Kawada et al., 2010). The expression and function of these peptides, however, have been studied only at the adult stage, and little is known about their expression during embryogenesis and larval formation.

In this study, we used a transgenic *Ciona* line and microarray analysis to isolate and characterize genes that are expressed in the *Ciona* larval brain, with an emphasis on genes encoding neuropeptides and hormone peptides. Our analysis identified 565 genes that were preferentially expressed in the larval brain. Among these genes, 11 encoded neuropeptides or hormone peptides, six of which had not been previously described. Receptors for some of the peptides were also expressed in brain tissues. Whole-mount *in situ* hybridizations showed the expression of peptide genes at the tailbud embryo stage. Interestingly, most of these genes were expressed in the ventral brain. These results suggest the existence of a neurosecretory region in the ascidian larval brain that is homologous to the vertebrate hypothalamus.

Materials and methods

Biologic materials

Wild-type *Ciona intestinalis* were obtained from the National BioResource Project in Japan (Department of Zoology, Kyoto University). Eggs and sperm were obtained surgically from the gonoduct. After insemination, eggs were dechorionated by immersing them in seawater containing 1% sodium thioglycolate (Wako Pure Chemical Industries, Osaka, Japan) and 0.1% actinase E (Kaken Pharmaceutical Co., Tokyo, Japan). After the embryos were washed with seawater, they were maintained in agar-coated dishes containing Millipore-filtered seawater and 50 μg/ml streptomycin sulfate (MFSW/S) at room temperature (18–20 °C). Tailbud embryos and tadpole larvae developed after 11 hrs and 17 hrs, respectively.

To isolate brain vesicles, we used a transgenic line that carried *Ci-β-tubulin* promoter (4.3 kb fragment of the 5′ up stream of *tubulin-β-2* [JGI gene model ID: ci0100135737])*::Kaede* (Horie et al., 2011). This line exhibits intense Kaede expression in the CNS of late tailbud embryos and larvae (Fig. 1A). After dechorionation, eggs from wild-type animals were fertilized using sperm from *Ci-β-tubulin (promoter)::Kaede* animals. Fertilized eggs were allowed to develop until the tailbud stage (13–15 hrs), and collected by hand centrifugation. Cells were dissociated by pipetting the specimens gently in Ca^{2+}–Mg^{2+}-free seawater with 0.1% trypsin. Embryonic cells began to dissociate within minutes. Dissociated cells were washed twice with Ca^{2+}–Mg^{2+}-free seawater containing 0.05% BSA and distributed over gelatin-coated Petri dishes. Using these dissociation conditions, Kaede-expressing brain cells were not completely isolated but rather formed clusters (Fig. 1B). Kaede-positive cell clusters and Kaede-negative control cells were collected with hand-made glass micropipettes.

Microarray analyses

Total RNA was extracted using the acid guanidium thiocyanate–phenol–chloroform method (Chomczynski and Sacchi, 1987). Aliquots of total RNA isolated from Kaede-positive or Kaede-negative cells (control samples) were labeled with either Cyanine-3 CTP or Cyanine-5 CTP (Perkin-Elmer/NEN Life Sciences, Boston, MA, USA) in a two-round linear amplification reaction using a Low RNA Input Fluorescent Linear Amplification Kit (Agilent Technologies, Palo Alto, CA). The quality and size distribution of the targets were determined in a RNA 6000 Nano Laboratory-on-a-chip assay (Agilent Technologies) and quantified using a NanoDrop microscale spectrophotometer (NanoDrop Technologies, Rockland, DE, USA). A set of fluorescently labeled cRNA targets (1 μg for each sample) was employed in a hybridization reaction with the *Ciona* oligoarray using an In Situ Hybridization Kit Plus (Agilent Technologies). A *Ciona intestinalis* 44-K custom-made oligo DNA microarray chip (Agilent Technologies; NCBI GEO Accession No. GPL5576) was used for comparative analysis with two color detection. The chip contains 42,034 oligonucleotide probes representing 19,964 genes. Hybridized microarrays were washed according to the manufacturer's protocol and then scanned on an Agilent Technologies G2565BA microarray scanner system with SureScan technology. The intensity of labeling was extracted from scanned microarray images using Feature Extraction 10.5 software (Agilent Technologies). All algorithms and parameters used in this analysis were from the software's default conditions (Yamada et al., 2005). Some probes that could not be assessed were excluded from the following analysis. Dye-swap analysis was carried out to reduce false-positive data. The data series have been deposited at NCBI GEO under the accession number GSE18364.

Similarity searches

The microarray probes represented established gene models and ESTs (Satou et al., 2005), which are accessible via NCBI GEO Accession No. GPL5576. Homology searches for the 565 genes were performed with tblastx algorithm using the NCBI human RefSeq mRNA database. Gene sequences that produced *P* values less than 10^{-5} were selected as homologs (described as “HS_BestHit” in the Tables). Genes are

Fig. 1. A *Ciona intestinalis* transgenic line that carries *Ci-β-tubulin(promoter)::Kaede*. (A) A larva showing strong Kaede expression in the CNS. (B) A cluster of Kaede-expressing cells. These clusters were collected and used to identify genes that are preferentially expressed in the larval brain. Scale bar, 100 μm.

denoted with gene collection Cluster IDs from the CLSTRID, the gene indices of The Institute for Genomic Research (TIGR), Kyoto Grailexp Gene 2005, JGI Gene v1, or JGI Gene v2. "GeneID" in the Tables refers to the serial number for each gene corresponding to the probes on the microarray.

To identify neuropeptides and hormone peptides, we looked for signal peptides in each deduced amino-acid sequence using SignalP 3.0 (http://www.cbs.dtu.dk/services/SignalP/) and an absence of any known functional protein domains using InterProScan Sequence Search (http://www.ebi.ac.uk/Tools/InterProScan/). Subsequently, typical dibasic endoproteolytic sites were manually detected in the sequences.

Whole-mount in situ hybridization

Specimens were fixed in 4% paraformaldehyde, 0.1 M MOPS (pH 7.5), and 0.5 M NaCl at 4 °C for 16 hrs, and stored in 80% ethanol at −30 °C. Preparation of RNA probes, washing of specimens, proteinase K treatment, postfixation, prehybridization, hybridization, and colorimetric reactions were carried out as described previously (Hamada et al., 2007).

Quantitative RT-PCR

Total RNA was prepared from Kaede-positive brain vesicle cells and Kaede-negative control cells. Total RNA (100 ng) was reverse transcribed using the SuperScript III first-strand synthesis system for qRT-PCR (Invitrogen). Real-time PCRs were performed using Power SYBR Green PCR Master Mix and an ABI Prism 7,500 system based on the manufacturer's protocol (Applied Biosystems). These experiments were repeated twice with different batches of larvae. Relative expression values were calculated by comparing the levels of expression in the brain and control cells. Values were normalized using the expression of GAPDH. All control samples lacking cDNA did not produce PCR products. Dissociation curves were used to confirm that single PCR products were amplified. The primers used in the present study are described in Supplementary Table S1.

Results

Isolation and characterization of genes that are preferentially expressed in Ciona larval brain

Transposon-mediated germline transgenesis of *Ciona intestinalis* has been described previously (Sasakura et al., 2003). To isolate brain vesicles, we used a transgenic line that carried *Ci-β-tubulin(promoter)::Kaede*. This transgenic line develops normally, undergoes metamorphosis, and grows up to become normal adult sea squirts. The line exhibited intense Kaede expression in the CNS of late tailbud embryos and larvae (Fig. 1A). When tailbud embryos were subjected to mild dissociation conditions, Kaede-expressing brain cells were not completely isolated but instead clustered in groups of approximately 100 cells (Fig. 1B). Considering this cell number and cluster size, the cluster would contain almost all cells of the brain vesicle (Cole and Meinertzhagen, 2004). In contrast, Kaede-expressing cells from other CNS regions completely dissociated. Kaede-positive clusters and Kaede-negative cells were collected, and total RNA was extracted. Cy3/Cy5-labeled total RNA was competitively hybridized to an oligo-based microarray to identify genes that are preferentially expressed in *Ciona* larval brain.

Analyses using a cut-off value of 3 and 2—namely, mRNA levels that were three and two times greater in experimental samples than in control samples—identified 286 and 565 candidate genes, respectively (see "Ratio" in the associated Tables). Hereafter, we refer to the 565 genes as the *Ciona* larval brain genes, all of which are listed in Supplementary Table S2. The 565 brain genes included 72 genes that have been examined brain expression in previously published studies and genes in such *Ciona intestinalis* gene databases as Ghost (http://hoya.zool.kyoto-u.ac.jp/cgi-bin/gbrowse/kh/) and Aniseed (http://aniseed-ibdm.univ-mrs.fr/) (Table S2).

The 30 genes associated with the highest expression ratios were further examined (Table 1). This group included previously reported *Ci-opsin1* (Kusakabe et al., 2001), *Ci-tyrosinase-related protein 1* (*Ci-TYRP1*) (Tassy et al., 2010), *Ci-tyrosinase* (Caracciolo et al., 1997), and *Ci-opsin3* (Nakashima et al., 2003; Mochizuki et al., 2003). Genes associated with Cluster IDs CLSTR04981 (Kusakabe et al., 2002), 06713 (Mochizuki et al., 2003), 00975 (Mochizuki et al., 2003), 14021 (Tassy et al., 2010), and 03272 (Mochizuki et al., 2003) were also identified in this group (Table 1). We used whole-mount *in situ* hybridizations to examine the expression of the other 21 genes in the larval brain. CIYS4168, 3970, 19345, and 2041 were not further analyzed because the probes for these genes were designed by the gene models but not supported by ESTs. Thus cDNAs of these genes could not be identified. We examined the expression of the remaining 17 genes at the late tailbud stage. One gene, CLSTR32977, did not produce a detectable hybridization signal. Nevertheless, the cDNA clone ID cinc004n14 revealed that this clone was originally isolated from a cDNA library of the adult neural complex, suggesting that it is expressed in the nervous system (Satou et al., 2005). The other 16 genes were detected in the brain, and some were expressed in the ganglion. Fig. 2 shows the results for CLSTR09578 (Fig. 2A), 00557 (Fig. 2B), 06749 (Fig. 2C), 36043 (Fig. 2D), and 14373 (Fig. 2E) as examples. Together, the results demonstrate that the identified genes are expressed preferentially in the *Ciona* larval brain.

Transcription factor and signal transduction genes

The *Ciona intestinalis* genome has been comprehensively annotated for transcription factor genes (Satou et al., 2003; Wada et al., 2003; Yagi et al., 2003; Yamada et al., 2003, Imai et al., 2004). We searched the 565 candidate genes for transcription factor genes and identified *Ci-BarH* (Imai et al., 2004), *Ci-Bsh* (Imai et al., 2004), *Ci-Prop* (Imai et al., 2004), *Ci-ONECUT1* (*one cut homeobox 1*) (Satou et al., 2001; Sasakura and Makabe, 2001), *Ci-Lhx* (Imai et al., 2004), *Ci-Rx* (D'Aniello et al., 2006), *Ci-Not* (Satou et al., 2001), *Ci-Six3/6* (Imai et al., 2004; Moret et al., 2005), *Ci-Unc4-A* (*UNC homeobox*) (Imai et al., 2004), *Ci-Otx* (Wada et al., 1996; Hudson and Lemaire, 2001), *Ci-Pax3/7* (Wada et al., 1996), *Ci-en* (Imai et al., 2004), *Ci-FoxC* (Imai et al., 2004), *Ci-FoxP* (Imai et al., 2004), *Ci-PTFb* (Imai et al., 2004), *Ci-COE* (Imai et al., 2004), *Ci-MYT1* (Imai et al., 2004), *Ci-DMRT1* (Imai et al., 2004), and *Ci-small optic lobes homolog-like* (Imai et al., 2004). With the exceptions of *Ci-BarH* and *Ci-small optic lobes homolog-like*, each of these genes has been previously shown to be expressed in the brain (Table 2). The present study also demonstrated that an additional transcription factor gene, *Ci-Prox-A*, was expressed in the brain (Table 2). All of these transcription factor genes have vertebrate homologs that are expressed in neural tissues. In particular, *Bsx (Bsh)*, *Six6*, and *Prop* were shown to be expressed in the developing vertebrate pituitary gland and hypothalamus (Jean et al., 1999; Olson et al., 2003; Nogueiras et al., 2006).

Signal transduction genes have also been identified in the *Ciona* genome (Hino et al., 2003; Satou et al., 2003; Imai et al., 2004). We found eight signal transduction genes among the 565 candidate brain genes, including two FGF genes (*Ci-FGF4/5/6* and *Ci-FGF11/12/13/14*), a Notch signaling gene (*Ci-Fringe 1*), a Wnt signaling gene (*Ci-Dkk3*), and three Hedgehog signaling genes (*Ci-Hedgehog1*, *Ci-Hedgehog2*, and *Ci-Niemann-Pick Type C1*). CLSTR01719 likely encoded a brain-specific component of the Hedgehog signaling pathway, because the deduced protein contained a patched domain (Table 2). *Ci-Hedgehog2* expression has been detected in the trunk endoderm adjacent to the brain (Islam et al., 2010), Although this result may have reflected contamination of the sample, *Ci-Hedgehog2* is the only gene in the

Table 1
The top 30 genes with highest expression ratios in the brain of *Ciona intestinalis* larva.

GeneID	Ratio	CLSTRID	GeneModel	HS_BestHit	GeneName	References
CIYS11728	10.1	CLSTR09578	AV865001	kelch-like 10 (Drosophila) (KLHL10)		
CIYS4534	7.9	CLSTR00557	TC83059			
CIYS5300	7.7	CLSTR34536	BW122154	natriuretic peptide receptor B/guanylate cyclase B (atrionatriuretic peptide receptor B) (NPR2)		
CIYS7677	7.7	CLSTR13524	KYOTOGRAIL2005.78.20.1	natriuretic peptide receptor B/guanylate cyclase B (atrionatriuretic peptide receptor B) (NPR2)		
CIYS8227	7.0	CLSTR06749	TC100137	phosphodiesterase 9A (PDE9A), transcript variant 1		
CIYS17657	6.9	CLSTR04981	TC99395			Kusakabe et al., 2002
CIYS9880	6.8	CLSTR36043	BW127219			
CIYS8037	6.8	CLSTR06713	TC93628	rhodopsin (RHO)	*Ci-opsin1*	Kusakabe et al., 2001
CIYS9617	6.6	CLSTR02951	TC97089			
CIYS8366	6.5	CLSTR15204	BW161282			
CIYS8909	6.5	CLSTR00975	TC93545			Mochizuki et al., 2003
CIYS18365	6.2	CLSTR00975	BW120385			Mochizuki et al., 2003
CIYS4168	6.2		KYOTOGRAIL2005.26.42.1	solute carrier family 45, member 2 (SLC45A2), transcript variant 1		
CIYS9055	6.2	CLSTR10642	TC103216			
CIYS21461	6.2	CLSTR02501	ci0100131307	glucan (1,4-alpha-), branching enzyme 1 (GBE1)		
CIYS17381	6.1	CLSTR14021	TC108247	tyrosinase-related protein 1 (TYRP1)	*TYRP1*	Tassy et al., 2010
CIYS8745	6.0	CLSTR32429	KYOTOGRAIL2005.761.2.2	glutamate decarboxylase 1 (brain, 67 kDa) (GAD1), transcript variant GAD67		Tassy et al., 2010
CIYS19329	5.9	CLSTR03272	TC100963	retinaldehyde binding protein 1 (RLBP1)		Mochizuki et al., 2003
CIYS7042	5.9	CLSTR32977	TC88788			
CIYS6558	5.7	CLSTR14634	TC83630		*Ci-Tyrosinase*	Caracciolo et al., 1997
CIYS7842	5.6	CLSTR32248	TC101983	dopachrome tautomerase (dopachrome delta-isomerase, tyrosine-related protein 2) (DCT), transcript variant 1		
CIYS3434	5.6	CLSTR01143	KYOTOGRAIL2005.333.15.1	retinal G protein coupled receptor (RGR), transcript variant 2	*Ci-opsin3*	Mochizuki et al., 2003
CIYS3970	5.6		KYOTOGRAIL2005.30.73.1	cyclic nucleotide gated channel alpha 1 (CNGA1), transcript variant 2		
CIYS4735	5.5	CLSTR04924	TC102640	synaptotagmin III (SYT3)		
CIYS19345	5.5		TC103061	natriuretic peptide receptor B/guanylate cyclase B (atrionatriuretic peptide receptor B) (NPR2)		
CIYS19381	5.4	CLSTR14373	TC80441			
CIYS18676	5.4	CLSTR06825	TC93813	PREDICTED: similar to phosphotidylinositol phosphatase PTPRQ precursor (Receptor-type tyrosine-protein phosphatase Q) (PTP-RQ)		
CIYS21535	5.4	Same to CIYS9617				
CIYS2041	5.4		NP1841292	BarH-like homeobox 1 (BARHL1)		
CIYS19501	5.3	CLSTR13675	TC97903	G protein-coupled receptor kinase 5 (GRK5)		

Nine of them have been reported by previous studies (references therein) to express in the larval brain.

transcriptional factor and signal molecule genes from which it has been shown to be expressed in the tissue other than the CNS. With the exception of *Ci-Dkk3*, none of the genes have been shown to be expressed in the larval brain, in part because their expression profiles were not always examined at late tailbud or larval stages.

Genes encoding neuropeptides and hormone peptides

We identified five previously reported peptides: the *Ciona*-specific GnRH family member t-GnRH-X (the corresponding gene is *Ci-gnrh-X*; Tello et al., 2005), the GnRH family member t-GnRH-3,5,6 (*Ci-gnrh-1*; Adams et al., 2003), the calcitonin family peptide Ci-CT (*Ci-CT*; Sekiguchi et al., 2009), the oxytocin/vasopressin family peptide Ci-VP (*Ci-VP;* Kawada et al., 2008), and the tachykinin family peptide Ci-TK-1 (*Ci-TK*; Satake et al., 2004; Aoyama et al., 2008) (Table 3). In both vertebrates and ascidians, GnRH plays a critical role in reproduction (Terakado, 2001). *Ci-gnrh-1* and *Ci-gnrh-X* expression have been detected at embryonic and larval stages by RT-PCR and at the adult stage by RT-PCR and *in situ* hybridization (Adams et al., 2003; Kawada et al., 2009). Calcitonin plays a vital role in calcium metabolism in mammals (Wimalawansa, 1997). Vasopressin and oxytocin are structurally related peptides composed of nine amino acids. In vertebrates, oxytocin/vasopressin mediate osmoregulation and the contraction of smooth muscle (Balment et al., 2006: Goldstein, 2006). The multifunctional tachykinin peptides are potent vasodilators that also induce smooth muscle contractions in vertebrates. Ci-TK promotes oocyte growth in the *Ciona* ovary (Satake et al., 2004; Aoyama et al., 2008). The expression of *Ci-CT*, *Ci-VP*, and *Ci-TK* have previously been examined only in *Ciona* adults (Satake et al., 2004; Kawada et al., 2008; Sekiguchi et al., 2009), yet we found that these are genes predominantly expressed in the larval brain.

We also identified nine candidate genes that appeared to encode neuropeptides or hormone peptides. As shown in Fig. 3, the polypeptides encoded by CLSTR35053 (Fig. 3A), 00975 (Fig. 3B), 10700 (Fig. 3C), 12486 (Fig. 3D), 00939 (Fig. 3E), 16011 (Fig. 3F), 04981 (Fig. 3G), 06757 (Fig. 3H), and 15482 (Fig. 3I) harbored a signal peptide and peptide-like sequences flanked by typical dibasic endoproteolytic sites (Steiner, 1998). Otherwise, CLSTR10700, 12486 and 00939 contain transmembrane domains. Thus these genes were excluded from the peptide candidate genes (Table 3). CLSTR04981 (Kusakabe et al., 2002) and CLSTR00975 (Mochizuki et al., 2003) were previously shown to be expressed in the larval brain. CLSTR04981 likely encoded a galanin/galanin-like peptide because a completely conserved galanin/galanin-like peptide consensus motif (GWTLNS) was identified in the putative peptide sequence (Ohtaki et al., 1999; Lang et al., 2007) (Fig. 3G). In vertebrates, galanin is widely distributed in the mammalian nervous system and gut, where it modulates a diverse set of physiologic functions (Mechenthaler, 2008). In the brain, the highest galanin concentrations have been observed in the hypothalamus (López et al., 1991). CLSTR00975 potentially belongs to Neuropeptide Y/YY family, because a C-terminal Gly amidation signal immediately before a KR site and several amino-acid residues typical of the vertebrate Neuropeptide Y/YY superfamily were identified in the putative peptide sequence (Sheikh, 1991; Merkler, 1994) (Fig. 3B). In vertebrates, this neuropeptide is distributed in the various tissues, including gut, pancreas,

Fig. 2. Expression of five genes that were identified as *Ciona* larval brain genes. (A) CLSTR09578. (B) CLSTR00557. (C) CLSTR06749. (D) CLSTR36043. (E) CLSTR14373. All of the genes were expressed specifically or predominantly in the brain of late tailbud embryos. Arrows indicate hybridization signals in the brain.

and CNS, where it is responsible for a range of biologic activities (Tatemoto et al., 1982). No homologous sequences were found for the other *Ciona* peptide candidates.

Receptor genes

Next, we searched for candidate genes that encoded receptors, including those for neuropeptides, hormone peptides, and neurotransmitters, resulting in the identification of 38 genes (Table 4). Among these genes, we found GPCR genes that resembled those encoding neuropeptide and hormone peptide receptors, including GnRH receptor *Ci-GnRHR4* (Tello et al., 2005), tachykinin receptor *Ci-TKR* (Satake et al., 2004; Aoyama et al., 2008), oxytocin/vasopressin receptor *Ci-VPR* (Kawada et al., 2008), and calcitonin receptor *Ci-CTR* (Sekiguchi et al., 2009). Thus, we detected the expression of not only peptide ligands, but also their corresponding receptors in the brain (Tables 3 and 4), suggesting that these signaling systems were functioning within the developing *Ciona* brain. Four GnRH receptor genes (*Ci-GnRHR1-4*) and three GnRH peptide genes (*Ci-GnRH1*, *GnRH2*, and *GnRHX*) had been previously identified (Kusakabe et al., 2003; Adams et al., 2003; Tello et al., 2005). The present study showed that *GnRH-1*, *GnRH-X*, and *GnRHR4* were expressed in the brain. *Ci-CT* and *Ci-CTR* are present as single copies in the *Ciona* genome (Sekiguchi et al., 2009). *Ci-TK*, *Ci-TKR*, *Ci-VP*, and *Ci-VPR* are also single-copy genes, and the selectivity of the interactions between the respective ligands and receptors has been confirmed (Satake et al., 2004; Kawada et al., 2008).

Referring to the phylogenic analysis by Kamesh et al (2008), we found two putative galanin receptor-like genes and two putative glucagon-like peptide receptor (GLPR) genes. We also found seven genes that belong to the chemokine receptor cluster. Two of them are somatostatin and angiotensin-like peptide receptor (SALPR) like, and have similarity to human opioid receptor genes. In LDLRR/GLHR/LGR (low-density lipoprotein receptor repeat/Leucine rich repeat containing GPCR) subfamily, we found a LDLRR-GPCR gene encoding protein with INSL3/Relaxin binding GPCR-like transmembrane region and a gene classified to unclassified LGR-like gene (Kamesh et al., 2008).

Opsins are visual cycle GPCRs in photoreceptor cells. We detected the expression of three opsin genes: *Ci-opsin1*, *Ci-opsin3*, and a gene that was similar to human *opsin 4* (*OPN4*). *Ci-opsin1* expression was previously detected in photoreceptor cells of the ocellus (Kusakabe et al., 2001), whereas *Ci-opsin3* expression was shown to be widely distributed in the brain vesicle (Nakashima et al., 2003; Mochizuki et al., 2003).

We also identified a number of other putative GPCR genes, including *Ci-Nut* (Etani and Nishikata, 2002), unclassified adhesion-like gene that has similarity to mouse *G protein-coupled receptor 128* (*GRP128*), Tre 1/GPR84-like gene, another rhodopsin gene (Kamesh et al., 2008), and one each that was similar to human *leucine-rich-repeat containing G protein-coupled receptor 5* (*LGR5*) and *G protein-coupled receptor 155* (*GRP155*).

We examined the expression of six putative neurotransmitter receptor genes, including two that were similar to the gamma-aminobutyric acid (GABA) receptor gene *GABA receptor, pi* (*GABRP*); three that resembled the cholinergic receptor genes *cholinergic receptor, nicotinic, beta 2* (*neuronal*) (*CHRNB2*), *CHRNB4*, and *cholinergic receptor, muscarinic 3* (*CHRM3*); and one the was similar to the adrenergic receptor gene *adrenergic, alpha-2A-, receptor* (*ADRA2A*) (Table 4). Our results support previous studies describing brain GABA/glycinergic neurons, cholinergic neurons, and indicate a potential catecholaminergic transmission in the *Ciona* larval brain (Yoshida et al., 2004; Horie et al., 2008, 2009; Zega et al., 2008).

We identified seven new candidates for guanylyl cyclase receptor family genes. They included a gene that was similar to human *natriuretic peptide receptor A/guanylate cyclase A* (*atrionatriuretic peptide receptor A*) (*NPR1*) and three genes that resembled *natriuretic peptide receptor B/guanylate cyclase B* (*atrionatriuretic peptide receptor B*) (*NPR2*). Natriuretic peptides regulate fluid balance in vertebrates. The other guanylyl cyclase receptor family genes included *Ci-retinal guanylyl cyclase 2* (Tassy et al., 2010) and a gene that was similar to human *guanylate cyclase 1, soluble, beta 3* (*GUCY1B3*).

These results demonstrate that a number of neurotransmitter receptors, peptide receptors, and photoreceptors are predominantly expressed in the *Ciona* larval brain, indicating that the *Ciona* brain contains certain cells that require ligand–receptor interactions to perform their signal transduction functions.

Quantitative RT-PCRs for peptide and GPCR genes

The larval brain expression profiles of the genes encoding neuropeptides and GPCRs were confirmed using quantitative RT-PCRs (qRT-PCRs). Six genes encoding new peptide candidates, five encoding previously reported peptides, and 12 that were known as peptide–binding GPCR genes and putative GPCR genes were assessed (Tables 3 and 4). We again collected Kaede-positive and Kaede-negative cells, and extracted total RNA. The relative expression levels were calculated by comparing mRNA levels in the brain cells and other cells. The obtained values were normalized based on results for *GAPDH* gene expression.

In addition, *Ci-opsin1* expression was also examined as positive control, whereas *Ci-Cdx* and *Hox12*, which are expressed in the tail,

Table 2
Transcription factor genes and signaling molecule genes, characterized as *Ciona* larval brain-genes in the present study.

GeneID	Ratio	CLSTRID	GeneModel	GeneName	Expression in brain	References
Transcriptional Factors						
Homeobox genes						
CIYS2041	5.4		NP1841292	*BarH*	no	Imai et al., 2004
CIYS6551	4.9	CLSTR14785	TC99808	*Bsh*	Yes	Imai et al., 2004
CIYS17558	4.6	CLSTR33160	TC79314	*Prop*	Yes	Imai et al., 2004
CIYS4528	4.3	CLSTR00604	TC78692	*one cut homeobox 1 (ONECUT1)*	Yes	Satou et al., 2001; Sasakura and Makabe, 2001
CIYS7679	4.0	CLSTR32188	TC87278	*Lhx1*	Yes	Imai et al., 2004
CIYS2305	3.9	CLSTR45268	KYOTOGRAIL2005.84.23.1	*Rx*	Yes	D'Aniello et al., 2006
CIYS13945	3.8	CLSTR00380	TC100097	*Not*	Yes	Satou et al., 2001
CIYS15005	3.4	CLSTR11446	BW450816	*Six3/6*	Yes	Imai et al., 2004, Moret et al., 2005
CIYS17549	3.2	CLSTR33191	TC86468	*Unc4-A*	Yes	Imai et al., 2004
CIYS15702	2.8	CLSTR02856	TC90021	*Otx*	Yes	Wada et al., 1996, Hudson and Lemaire, 2001
CIYS21710	2.7		TC85887	*Prox-A*	NA	
CIYS9917	2.4		estExt_fgenesh3_pg.C_chr_10p0193	*Pax3/7*	Yes	Wada et al., 1996
CIYS19269	2.2	CLSTR13509	TC79227	*en*	Yes	Imai et al., 2004
Fox genes						
CIYS8235	3.2	CLSTR32249	TC78808	*FoxC*	Yes	Imai et al., 2004
CIYS19453	2.4	CLSTR02755	TC97214	*FoxP*	Yes	Imai et al., 2004
bHLH genes						
CIYS8249	4.2	CLSTR32256	TC88929	*PTFb*	Yes	Imai et al., 2004
CIYS17729	2.9	CLSTR05032	TC78882	*COE*	Yes	Imai et al., 2004
Other TFs						
CIYS6496	3.4	CLSTR14759	TC78635	*myelin transcription factor*	Yes	Imai et al., 2004
CIYS18143	2.9	CLSTR03505	TC108261	*DMRT1*	Yes	Imai et al., 2004
CIYS13591	2.6	CLSTR35792	TC80279	*small optic lobes homolog-like*	no	Imai et al., 2004
Signaling molecules						
FGF signaling						
CIYS20178	3.1	CLSTR10181	TC91885	*FGF4/5/6*	no	Imai et al., 2004
CIYS15305	2.7	CLSTR15889	TC81447	*FGF11/12/13/14*	no	Imai et al., 2004
Hedgehog signaling						
CIYS17708	2.7	CLSTR33736	TC84365	*Hedgehog2*	no	Takatori et al., 2002; Imai et al., 2004, Islam et al., 2010
CIYS11892	2.3	CLSTR04262	TC78835	*Hedgehog1*	no	Takatori et al., 2002; Imai et al., 2004
CIYS4738	2.2	CLSTR01719	KYOTOGRAIL2005.136.41.1	*patched domain containing 1*	NA	
CIYS10346	2.4	CLSTR37145	TC84436	*Niemann-Pick Type C1*	no	Imai et al., 2004
Notch signaling						
CIYS15068	2.6	CLSTR30086	KYOTOGRAIL2005.501.8.1	*Fringe 1*	no	Imai et al., 2004
Wnt signaling						
CIYS15315	5.3	CLSTR06734	TC91788	*Dkk3*	Yes	Tassy et al., 2010

Table 3
Genes encoding hormonal and/or neuronal peptides, characterized as *Ciona* larval brain-genes in the present study.

GeneID	Ratio	CLSTRID	Family	GeneName	References
Known peptides					
CIYS15578	3.6	CLSTR10761	GnRH	*Ci-gnrh-X*	Tello et al., 2005
CIYS19381	5.4	CLSTR14373	GnRH	*Ci-gnrh-1*	Adams et al., 2003
CIYS8179	3.6	CLSTR32230	Calcitonin	*Ci-CT*	Sekiguchi et al., 2009
CIYS7042	5.9	CLSTR32977	Oxytocin/ Vasopressin	*Ci-VP*	Kawada et al., 2008
CIYS8008	4.4	CLSTR36631	Tachykinin	*Ci-TK*	Satake et al., 2004, Aoyama et al., 2008
New peptide candidates					
CIYS7997	3.7	CLSTR35053			
CIYS3465	3.1	CLSTR16011			
CIYS9446	5.3	CLSTR06757			
CIYS19731	4.0	CLSTR15482			
CIYS17657	6.9	CLSTR04981	galanin/ galanin-like peptide family		Kusakabe et al., 2002
CIYS8909	6.5	CLSTR00975			Kusakabe et al., 2002, Mochizuki et al., 2003

were used as negative controls (Kusakabe et al., 2001; Imai et al., 2004; Ikuta et al., 2004). As expected, both microarray and qRT-PCR analyses showed that *Ci-opsin1* was more highly expressed in the larval brain, whereas *Ci-Cdx* and *Ci-Hox12* expression levels were relatively low in the larval brain.

The qRT-PCR analyses clearly demonstrated that all 23 genes were predominantly expressed in the larval brain (Fig. 4, Supplementary Figure S1). The expression ratios of the genes determined by qRT-PCR analysis were similar or slightly higher than those obtained from the microarray analysis. For example, the lowest microarray ratio was 2.2 for CLSTR13071 (CIYS8277), whereas the qRT-PCR ratio for this gene was 2.4 (Fig. 4). These results suggested that a threshold value of 2 in the microarray analysis reliably identified genes that were specifically or preferentially expressed in the *Ciona* larval brain.

Spatial expression of peptide genes

The spatial expression profiles of the peptide and GPCR genes that were analyzed by qRT-PCR were further examined by whole-mount *in situ* hybridization at the mid-tailbud, late tailbud, and larval stages. We obtained positive results for nine peptide genes, whereas most of the GPCR genes failed to produce a hybridization signal.

The expression of *Ci-gnrh-X* (*Ciona* GnRH gene), which was first detected at the mid-tailbud stage (Fig. 5A left), was evident in the

ventral embryonic and larval brain (Fig. 5A). Another GnRH gene, *Ci-gnrh-1*, was expressed in the posterior brain at the late tailbud stage and was broadly expressed in the brain at the larval stage (Fig. 5B).

A. CLSTR35053
MNKSKLSRGGLGLKLGLVVVISLLVLATSVESRRRMSRMQLKE
FVCRRCWSKLYHRCYYGVGLSKRSISESEANYEPEASLEQTSL
VSQDTLDLFNGHQRTKRSAGRVRRSGRRRYYRFRINCHICQIN
CFR

B. CLSTR00975
MHVIKLVRFTLVVGCILMSGVQSYENSERPLNAQLQYEDEPYF
NSVDADVKYPENYLEELEEDILQQQKPTRFLNRDGAVKKRNHA
EGTMTDLKGRYYRYGLHQLRNDWIQDMINGRYGKRVNTRSPVY
DSVAYAPPSSNVDRELSDLSERFRNYLQWKQAYETLNRMYRSK
EEQKPESSNSE

C. CLSTR10700
MSMTLLLGSTGSIMLAIMAMRNSVFHVHRTVAGFSVYQQPEED
IPAAVVQHRTKFKAWRSKNPSKFALGLISVNWLVAILFLIVSL
SLQDCLATCECLFGFVSRSEMNVCKSPKATCLNKDPPMHANAV
ILSIVIWFLSVTSLVVILVRFGIDYKNFKFKVVESLRVKNNSV
ETASRNISSREDDLVVQNSAGRSLPTPERRFRAPTSMLLRSLR
RKILSLVFVSVIFGAGTGYREIRDLISFFQSILMKDTGTQHVL
NPFSSAIEIISSVVLCWVARGTQTALRSAFRAIIHALNCRPT

D. CLSTR12486
MSRCLQISSIVLALAAMIFVIVSFVTTSWMTACMDAKHILAMA
AAIVQIPDSAKAMFNGKICFDTGLFKVSYSFMNMHSAVDLPAN
SMDAFLKITIGLFSFSLLLLLVGGILIILAVCSVSMSSQRKAI
ARMYNGIGAVVLFFGAIFILAGALAYTVPQSRLVHQPLPWMAQ
LGKTFQNIMAGGLQNMPGSSSFSGLNNLGGHDGSLPSIPDLSH
LGGNSESSSSLPPGFGNLGNLDSSSLQHLGDMMGTGRKKRQIS
GMDSTMLQNLLGSIDPMFGYSLYLSYVAFIFALLSAIFAAVAS
RRGGGEYPDTVPLDTNFGKI*

E. CLSTR00939
MVTIKMSFLYLMTIVFASLYFCTAADETKEQPEQPEQPAVENT
ITEVKPEENEPKNATKVENVIPSVHNHTLDKVPGFFNMENNYP
MLKRGFYVMLVAMSLMMVYMVVKYYRTKKRKTKRYGLLGSKNE
NIELNALQSDSEDDYTVFEAKTFH

F. CLSTR16011
MKNIFLVSVLIFAYQSTTLADDTHSMNTRGRVDPRKEFCNELI
NGGRVGFSMIQQLCGSYGKRMFSRTRTTRNEPIVTSRRSGSAL
PEAMMSDDTFNTRGSYNGRKTSYRTGRRPEHELVSRLAAALRR
QFSSVTKHSNFDRPKDDWRVRDTDGPMYLIKKWLRYDA

G. CLSTR04981 (galanin/galanin-like peptide family)
MNSFGRYTFSLAVVLYISIVLCAENSEAATAKRPFRGQGGWTL
NSVGYNAGLGALRKLFEKRDGSSLDVESMPLDNEEMENLAKDF
ALFLEVKESGLLGPRMLRCILSRNDQVMDSMSEM

H. CLSTR06757
MKLVKSFSILAAFVVCYFGCIADAVPVDTVEKQLLRREGTGNP
ENFLDWTNQLNSTDVDETDEELYDQLLYNILIPMQQKMIAEEN
GQLDNAEDNPFLAENRNSDEENNDYSPGQAESIQSDKRFQSLF
KRYPGFQGLFKRHNPHLPDLFKRYNSMGLFKRSPGMLGLFKRG
LLGLFKRSDARLQGLFKRDSATQGSFKRSSEAQALPKRYPNFQ
GLFKRLSEATEYPEDDSSNDDTKQRGNLHSLFKRDTSAHYLED
RGESIPFLFRRS

I. CLSTR15482
MKLTFLFVVLMVLYDVTTAQDDVRDVINDEDIDRAIEVLSRLQ
EEETNPDLTDGDQKMEDTREKYDMEGEADGNDNKEERDPLTNI
MKRFYIKVVRQYCPPGQQCLGFRGRHRWQRCTCPGRTRCQKSP
GYGYECL

Interestingly, Ci-GnRH-X was previously shown to antagonize the effects of t-GnRH-6 at Ci-GnRHR-1 (Kawada et al., 2009). Therefore, the activity of Ci-GnRH1 may be inhibited in the Ci-GnRH-X-expressing region of the larval brain. In adults, *Ci-gnrh-1* and *Ci-gnrh-X* are reportedly expressed in neurons residing the brain ganglion. *Ci-gnrh-1* was coexpressed with *Ci-gnrh-X*, although *Ci-gnrh-X* was expressed alone in several other neurons (Kawada et al., 2009).

The expression of *Ci-VP* (oxytocin/vasopressin gene) was observed in the posterior ventral brain at the larval stage (Fig. 5C). In adult *Ciona*, RT-PCR and *in situ* hybridization analyses demonstrated robust expression of *Ci-VP* in several neurons from the neural ganglion (Kawada et al., 2008). *Ci-TK* (tachykinin gene), on the other hand, was expressed in the anterior ventral brain at the tailbud stage, an expression domain that expanded around the pigmented cells in the larval brain (Fig. 5D). In adults, RT-PCR analysis demonstrated that *Ci-TK* was expressed in neurons from the brain ganglion, intestine, and endostyle (Satake et al., 2004).

Among the newly-identified candidate peptide genes, we were able to detect the expression of CLSTRs 15482, 16011, 06757, 04981, and 00975. CLSTR04981 encodes a galanin/galanin-like peptide family gene, expression of which was observed in the ventral larval brain (Fig. 5F). CLSTR00975 was detected at the mid-tailbud stage in the ventral brain, whereas weak signals were observed in the anterior brain (Fig. 5E). CLSTR16011 was expressed in part of the nerve cord in mid-tailbud embryos and in the ventral brain in late tailbud embryos (Fig. 5G left; a while arrow), but was not detected at the larval stage (Fig. 5G right). CLSTR06757 was expressed in ventral and posterior regions of the brain at the mid-tailbud and late tailbud stages and additional expression was observed in some dorsal CNS cells at the larval stage (Fig. 5H; white arrows). CLSTR15482 was expressed in posterior ventral brain at the late tailbud stage (Fig. 5I).

Together, the results show that most of these genes were expressed in the ventral brain, but the specific expression domains were slightly different among the genes. We also found that these genes were expressed from the mid or late-tailbud stage.

Discussion

The present study used a transgenic *Ciona* line combined with microarray analysis to identify genes that are expressed in the larval brain. The resulting 565 candidate genes indicate that this is a powerful method to screen for genes that are preferentially expressed in a specific organ or tissue of *Ciona* larvae or adults. The specific or preferential expression of the identified genes in the brain was confirmed by our and previous *in situ* hybridization analyses; 25 of the 30 best candidate genes are preferentially expressed in the larval brain (Table 1). We focused on genes encoding peptides and found that a number of known neuropeptides and hormones, as well as new peptides were expressed in the *Ciona* larva brain. The results were confirmed using qRT-PCR and whole-mount *in situ* hybridizations.

Ciona larvae have served as a good model for brain development owing to the small number of constituent cells (~330 cells) and the well-documented positions of these cells in the CNS (Cole and Meinertzhagen, 2004; Imai and Meinertzhagen, 2007). The simple nervous system facilitates examinations of chordate neuroethology at the single-cell level using neuron-labeling strategies that have been widely adopted for other invertebrate systems (Meinertzhagen et al., 2004). The catalog of brain genes identified in this paper should lay

Fig. 3. Deduced amino-acid sequences of peptide candidate genes that harbored a signal peptide and peptide-like sequences flanked by typical dibasic endoproteolytic sites. (A) CLSTR35053. (B) CLSTR00975. (C) CLSTR10700. (D) CLSTR12486. (E) CLSTR00939. (F) CLSTR16011. (G) CLSTR04981 (galanin/galanin-like peptide family). (H) CLSTR06757. (I) CLSTR15482. Possible signal peptides are underlined, dibasic endoproteolytic sites are denoted in blue, predicted peptide sequences are shown in red, cysteine residues that may form disulphide bonds are marked in magenta, and C-terminal amidation signals are denoted in green.

Table 4
Receptor genes characterized as *Ciona* larval brain-genes in the present study.

GeneID	Ratio	CLSTRID	GeneModel	HS_BestHit	GeneName/Family	Reference
GPCRs						
CIYS988	4.4	CLSTR40102	TC87761	gonadotropin-releasing hormone receptor (GNRHR)	*Ci-GnRHR4*	Tello et al., 2005
CIYS3329	4.0	CLSTR15345	TC93544	tachykinin receptor 2 (TACR2)	*Ci-TKR*	Satake et al., 2004; Aoyama et al., 2008
CIYS9235/12665	4.3/ 3.5	CLSTR06650	TC88699	arginine vasopressin receptor 1B (AVPR1B)	*Ci-VPR*	Kawada et al., 2008
CIYS7545	4.4	CLSTR32782	TC81380	parathyroid hormone 1 receptor (PTH1R)	*Ci-CTR*	Deyts et al., 2006, Sekiguchi et al., 2009
CIYS13466	3.1	CLSTR34840	TC94802	galanin receptor 1 (GALR1)	GALANIN receptor like	Kamesh et al., 2008
CIYS8277	2.2	CLSTR13071	estExt_fgenesh3_pg. C_chr_04q0346	galanin receptor 1 (GALR1)	GALANIN receptor like	Kamesh et al., 2008
CIYS18623/15057	3.0/ 2.4	CLSTR16958	TC97403		GLPR-like	Kamesh et al., 2008
CIYS1556	2.7		ci0100140366	opioid receptor, kappa 1 (OPRK1)	SALPR like	Kamesh et al., 2008
CIYS2281	3.5		ci0100150689	opioid receptor, mu 1 (OPRM1)	SALPR like	Kamesh et al., 2008
CIYS12218	2.7	CLSTR32882	KYOTOGRAIL2005.13.22.1	neuropeptides B/W receptor 1 (NPBWR1)	unclassified Chemokine receptor cluster like	Kamesh et al., 2008
CIYS17897	3.4	CLSTR03282	TC81170	somatostatin receptor 2 (SSTR2)	unclassified Chemokine receptor cluster like	Mochizuki et al., 2003; Kamesh et al., 2008
CIYS538	2.6		ci0100133186	formyl peptide receptor 2 (FPR2)	unclassified Chemokine receptor cluster like	Kamesh et al., 2008
CIYS8024	3.0	CLSTR35041	TC92771	complement component 3a receptor 1 (C3AR1)	unclassified Chemokine receptor cluster like	Kamesh et al., 2008
CIYS19298/2479	4.5/ 2.8	CLSTR10714	TC92777		unclassified Chemokine receptor cluster like	Kamesh et al., 2008
CIYS3828/12260	3.8/ 3.1	CLSTR37142	TC87628	relaxin/insulin-like family peptide receptor 1 (RXFP1)	LDLRR-GPCR	Kamesh et al., 2008
CIYS2684	5.0		KYOTOGRAIL2005.12.33.1		unclassified LGR-like	Kamesh et al., 2008
CIYS8037	6.8	CLSTR06713	TC93628	rhodopsin (RHO)	*Ci-opsin1*	Kusakabe et al., 2001
CIYS3434	5.6	CLSTR01143	KYOTOGRAIL2005.333.15.1	retinal G protein coupled receptor (RGR)	*Ci-opsin3*	Nakashima et al., 2003; Mochizuki et al., 2003
CIYS876	3.4		ci0100136496	opsin 4 (OPN4), transcript variant 2		
CIYS10116/17319/ 21465	3.7/ 3.1/ 3.3	CLSTR02054	TC107849	ribosomal protein S10 (RPS10)	*CiNut*	Satou et al., 2001; Etani and Nishikata, 2002
CIYS10706	2.8	CLSTR36282	TC94073	G protein-coupled receptor 128 (Gpr128): Mouse	unclassified Adhesion-like	Kamesh et al., 2008
CIYS3922	3.2		ci0100154093	adrenergic, alpha-2A-, receptor (ADRA2A)	unclassified Amine Receptor-like	Kamesh et al., 2008
CIYS1098	5.0		KYOTOGRAIL2005.214.21.1	cholinergic receptor, muscarinic 3 (CHRM3)	Muscarinic Receptor like	Kamesh et al., 2008
CIYS18058	2.4		TC88375	PREDICTED: hypothetical LOC732377 (LOC732377)	Tre 1/GPR84 like	Kamesh et al., 2008
CIYS7936/ CIYS20	3.8/ 2.9	CLSTR14927	TC95482		Other Rhodopsins	Kamesh et al., 2008
CIYS16327	2.5	CLSTR09927	ci0100152288	G protein-coupled receptor 155 (GPR155)		
CIYS6449	2.1	CLSTR14825	TC91091	leucine-rich repeat-containing G protein-coupled receptor 5 (LGR5)		
Ionotropic receptors						
CIYS1233	3.6	CLSTR38510	TC97995	gamma-aminobutyric acid (GABA) A receptor, pi (GABRP)	*Ci-GlyR*	Tassy et al., 2010
CIYS3120	2.7		ci0100149121	gamma-aminobutyric acid (GABA) A receptor, pi (GABRP)		
CIYS1869	3.4		ci0100132435	cholinergic receptor, nicotinic, beta 2 (neuronal) (CHRNB2)	*Ci-nAChR-B/G/D/E1*	Tassy et al., 2010
CIYS3457	2.4		ci0100154594	cholinergic receptor, nicotinic, alpha 4 (CHRNA4)		
Guanylyl cyclase receptors						
CIYS7802	4.9	CLSTR00822	TC83864	natriuretic peptide receptor A/guanylate cyclase A (NPR1)		Satou et al., 2001
CIYS5300	7.7	CLSTR34536	BW122154	natriuretic peptide receptor B/guanylate cyclase B (NPR2)		
CIYS7677	7.7	CLSTR13524	KYOTOGRAIL2005.78.20.1	natriuretic peptide receptor B/guanylate cyclase B (NPR2)		
CIYS19345	5.5		TC103061	natriuretic peptide receptor B/guanylate cyclase B (NPR2)		
CIYS3486	5.3	CLSTR33422	KYOTOGRAIL2005.2118.1.1	natriuretic peptide receptor B/guanylate cyclase B (NPR2)		
CIYS8211	5.3	CLSTR14619	TC83639	guanylate cyclase 1, soluble, beta 3 (GUCY1B3)		
CIYS1684	4.3		KYOTOGRAIL2005.165.13.1	guanylate cyclase 2 F, retinal (GUCY2F)	*Ci-RETINAL GUANYLYL CYCLASE 2*	Tassy et al., 2010

the foundation for future neurobiology studies of chordates. Technical improvements may also extend our work; for instance, complete dissociation of embryonic cells should allow collection of Kaede- or GFP-positive cells using a cell sorter, providing a more efficient method for sampling of target cells from other tissues in future studies (Christiaen et al., 2008). Although we could detect the genes that were known to express in the brain and those newly identified in this study, it should be mentioned that all the genes expressed in the brain were not always able to be identified by this microarray analysis. For example, our method might be unable to detect genes that have

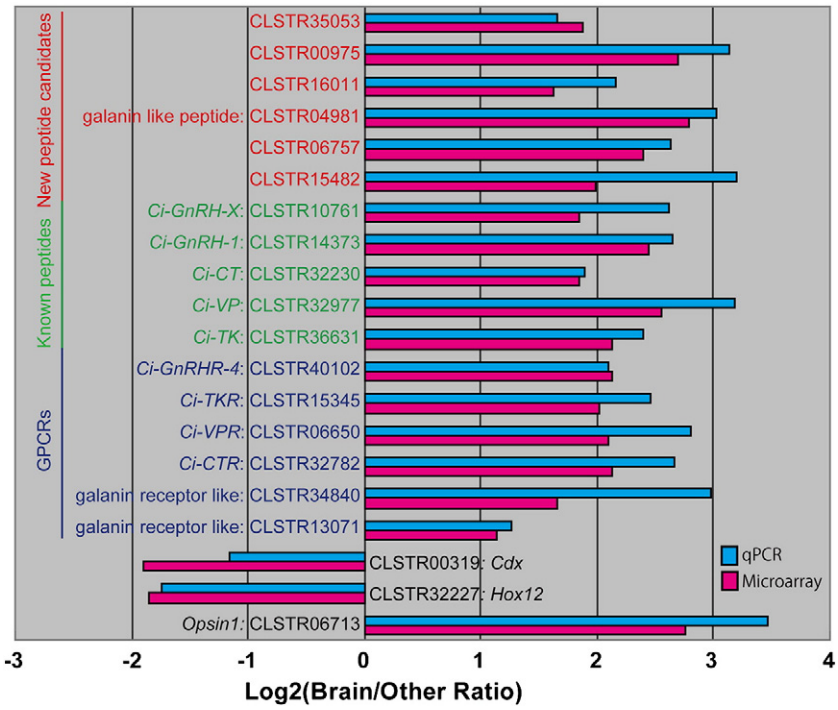


Fig. 4. qPCR analysis of peptide genes and peptide-binding GPCR genes. The horizontal axis shows the ratio (log2) of the mRNA expression levels in the brain to those in other regions. The vertical axis shows the examined gene. *Ci-Cdx* and *Ci-Hox12*, which are expressed in the larval tail, were used as negative control samples, whereas *Ci-Opsin1* was used as positive control brain gene.

strong expression in other tissues too, those of which expression quantity is excessively low, or those of which probe does not match to the actual mRNA sequence.

Peptide genes and receptor genes

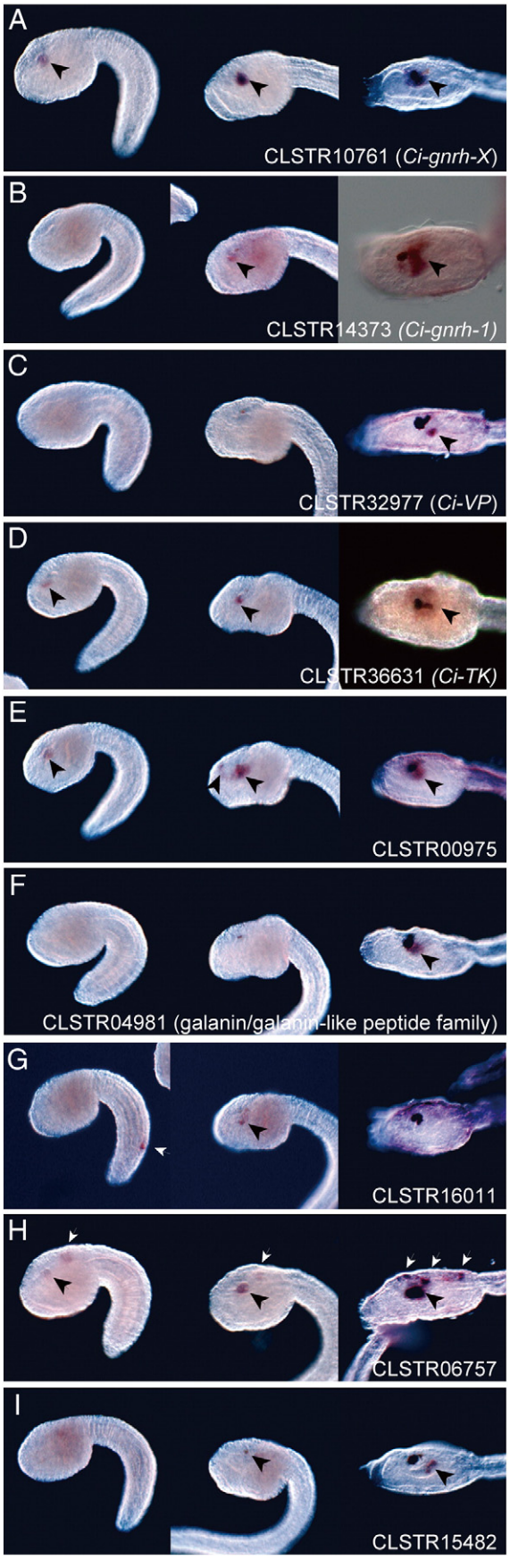
The present study uncovered an unexpected number of genes encoding peptide ligands and their corresponding receptors, including calcitonin (*Ci-CT*; Sekiguchi et al., 2009) and its receptor Ci-CTR (Sekiguchi et al., 2009); GnRH (*Ci-GnRH1, Ci-GnRHX*; Adams et al., 2003; Tello et al., 2005; Kawada et al., 2009) and its receptor GnRHR (*Ci-GnRHR4*; Tello et al., 2005); oxytocin/vasopressin (*Ci-VP*; Deyts et al., 2006; Kawada et al., 2008) and its receptor Ci-VPR (Kawada et al., 2008); tachykinin (*Ci-TK*; Satake et al., 2004; Aoyama et al., 2008) and its receptor Ci-TKR (Satake et al., 2004; Aoyama et al., 2008); and galanin/galanin-like peptide (CLSTR04981) and potential receptors (CLSTR34840 and 13071) (Fig. 4). Previous studies have paid less attention to the expression of these genes in the larval brain, probably because the ascidian larvae possess no functional circulation, digestion, or reproduction systems. On the other hand, the expression and function of some peptides have been investigated in these systems in the adult ascidian. For example, Aoyama et al. (2008) reported that tachykinin, a key player in oocyte growth, is expressed in the adult neuronal complex, whereas its receptor is expressed in the test cells that surround young oocytes. On the other hand, the expression of tachykinin and its receptor in the larval brain had not been examined (Satake et al., 2004; Aoyama et al., 2008).

Peptide hormones act not only as endocrine agents but also as paracrine/autocrine factors to regulate cell differentiation during development. For example, neuropeptides are essential for neuronal differentiation in hydra (Bosch and Fujisawa, 2001) and development of reproductive system in planarian (Collins et al., 2010). In vertebrates, several peptides such as growth hormone and thyroid hormone and their receptors are synthesized in developing tissues where they function locally to regulate tissue differentiation (Flamant and Samarut, 1998; Harvey et al., 2001; Tata, 2006; Sanders and Harvey, 2008). The present microarray and qPCR analyses addressed the expression of both ligands and receptors in the *Ciona* larval brain, suggesting that these proteins interact and function within the brain in a paracrine/autocrine manner. Although we have not yet obtained functional data, we expect that these peptides are involved in regulating osmotic pressure, sensing of environmental cues, promoting larval movement, and/or initiating metamorphosis. Of note, some peptide genes were expressed during development of tailbud embryos (Fig. 5 and Fig. 6), suggesting a possibility of some roles in differentiation of the larval brain cells.

Alternatively, peptide-synthesizing neurons (or their precursors) in the larval brain may be prepared for metamorphosis, after which newly produced peptide mRNAs are used by the adult. The ascidian larval nervous system is re-constructed during metamorphosis as the neural complex forms in the adult (Nicol and Meinertzhagen, 1991; Satoh, 1994; Manni et al., 1999, 2005; Dufour et al., 2006). Recently, it has been shown that most parts of the larval CNS are maintained during metamorphosis and recruited to form the adult CNS. Especially, some of ependymal cells of the larval CNS differentiate into neurons in the adult CNS (Horie et al., 2011). The mechanism that govern the development of adult neural tissue have not yet been fully clarified, however. Elucidating the functions of the genes identified in the present study should facilitate analyses of adult neural tissue development, and is thus an intriguing subject for future studies.

Evolutionary link between the ascidian larval brain and vertebrate hypothalamus

The present results also support an evolutionary link between the ascidian larval brain and vertebrate hypothalamus. Although only vertebrates, including basal vertebrates hagfish and lamprey, have a distinct hypothalamus, cell clusters that function as the neurosecretory center are found in the forebrain of many animals including annelids, mollusks and insects (Hartenstein, 2006; Tessmar-Raible et al., 2007). In basal chordates, the neural gland in the neural complex of adult ascidians and Hatschek's pit in the oral cavity of amphioxus are thought to exhibit some homology with the vertebrate hypothalamus based on morphologic characteristics and the expression of such hypothalamic peptides as



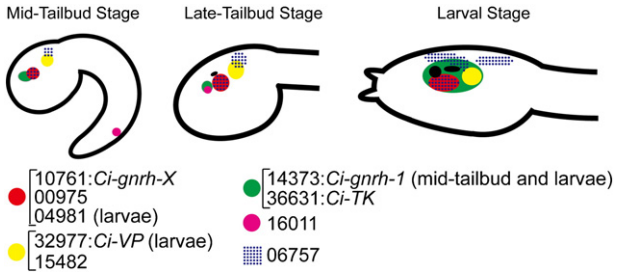


Fig. 6. Summary diagram of the expression pattern of peptide genes. The expression profiles of CLSTR10761 (red), 00975 (red), 04981 (red), 32977 (yellow), 15482 (yellow), 14373 (green), 36631 (green), 16011 (magenta), and 06757 (blue dots) at the mid-tailbud stage (left), late-tailbud stage (center), and larval stage (right) are shown. The numbers indicate CLSTR_ID. In case expression signals of the genes were observed at not all the stage, the parentheses indicate the stages at which expression was observed.

GnRH (Chang et al., 1985; Adams et al., 2003; Tello et al., 2005; Kozmik et al., 2007; Kawada et al., 2008; Nozaki, 2008; Sower et al., 2009; Terakado, 2009). Ascidians, the closest relative of vertebrates, would provide important hints of the evolution from invertebrate neurosecretory center to vertebrate hypothalamus-pituitary system. However, it is not easy to compare the structural similarity and differentiation process of the neural grand of ascidians to the other animals because of unique and diverged body plan of the adult ascidian. Moret et al. (2005) examined the expression of transcription factor genes involved in vertebrate forebrain patterning and found that the brain vesicle of *Ciona* larvae contains a domain that appears to correspond to the vertebrate presumptive hypothalamus. The present study revealed the expression of other hypothalamus-related genes in the *Ciona* larval brain, namely, such transcription factor genes as *Bsh* and *Prox*, and such hormone peptide genes as *GnRH* and *VP*. These results support the existence of a region in the ascidian larval brain that is homologous to the hypothalamus.

This idea was further supported by the expression of these peptide genes in a specific domain in the brain. Moret et al. (2005) suggested that the vertebrate hypothalamus corresponds to a ventral region in the *Ciona* larval brain vesicle on the basis of the expression of the hypothalamic transcriptional factors such as *Otp*, *Meis and Nkx2.1*. Our data showed that many peptide genes, including those encoding Ci-GnRH-X, Ci-VP and newly identified peptides, were expressed in the ventral brain (Fig. 5 and Fig. 6). These results strongly suggest that the ventral region of the *Ciona* brain is a hypothalamic domain and its structure would be taken over as the vertebrate pituitary-hypothalamus system.

For further understanding evolutional link of hypothalamic region between vertebrate and ascidian, we need comparative analyses of the cell lineage, the differentiation process and the gene regulatory cascades of the hypothalamic cell. In the development of vertebrates, GnRH neurons and VP/OT neurons come from different tissues and migrate to the definitive regions. GnRH neurons appear in the olfactory placode and subsequently migrate into the preoptic area of the anterior hypothalamus during embryonic development (Schwanzel-Fukuda and Pfaff, 1989, Wray et al., 1989). Otherwise, VP/OT neurons originate in the neuroepithelium and migrate to the supraoptic and paraventricular nuclei of the anterior hypothalamus (Shimada and Nakamura, 1973; Altman and Bayer, 1978). For the differentiation of VP/OT neurons, the expression of *Otp and Nk2.1* is required (Kimura et al., 1996; Acampora et al., 1999). The region expressing these transcriptional factor genes is overlapping and also including the lineage of VP/OT

Fig. 5. Expression of neuropeptide and hormonal peptide genes in the brains of *Ciona intestinalis* larvae. (A) CLSTR10761 (*Ci-gnrh-X*). (B) CLSTR14373 (*Ci-gnrh-1*). (C) CLSTR32977 (*Ci-VP*). (D) CLSTR36631 (*Ci-TK*). (E) CLSTR00975. (F) CLSTR04981 (galanin/galanin-like peptide family). (G) CLSTR16011. (H) CLSTR06757. (I) CLSTR15482. The expression patterns at the mid-tailbud stage (left), late tailbud stage (center), and larval stage (right) are shown for each gene. Black arrows indicate hybridization signals in the brain. White arrows indicate the signals outside of the brain.

neurons (Tessmar-Raible et al., 2007). In contrast, *Ciona Otp, Meis,* and *Nkx2.1* are expressed in consecutive anteroposterior territories of the ventral brain at the tailbuds stage (Moret et al., 2005), and *Ci-VP* was expressed more posteriorly (Fig. 5 and Fig. 6). Lineage data suggest the cells expressed these transcriptional factors are derived from the a8.19 and a8.17 pairs of blastomeres (Nishida, 1987; Cole and Meinertzhagen, 2004; Moret et al., 2005). However it is not known from which blastomeres VP-expressing cells are derived.

In addition to the present *in situ* hybridization analysis, Shimozono et al. (2010) showed that the spatial expression profiles of various brain-specific genes were not identical but instead revealed various distinct compartments in the brain. Of note, the regional expression of the *Ciona* brain genes is not always defined by their cell lineage, suggesting the presence of regulatory mechanisms that control the regionalized expression of the brain-specific genes. Future studies should delineate more precise expression profiles of the genes at the single-cell level to discrete areas in the ventral brain-vesicle region of the *Ciona* larva.

Brain architecture and gene expression

Previous studies identified at least five different types of organs and/or tissues in the ascidian brain: the ocellus, otolith, presumed hydropressure receptors, sensory vesicle cavity, and neurohypophysis (Meinertzhagen et al., 2004). In addition, approximately 100 neurons, including glutamatergic, cholinergic, GABA/glycinergic, serotoninergic, and catecholaminergic neurons, are present in the ascidian brain structures (reviewed by Horie et al., 2009). The genes identified in this study likely reflect the complexity of the ascidian brain. For example, a number of neurotransmitter receptor genes are expressed in the ocellus, whereas *Ci-AMT* (*Ammonium Transporter*) is expressed in the otolith (Caracciolo et al., 1997; Nakagawa et al., 2002; D'Aniello et al., 2006; Marino et al., 2007). Although the present study identified various genes that were previously known to be expressed in different tissues or cells, many of the newly identified peptides and receptors require further analysis to determine their expression patterns and functions. These genes may be involved in brain development or functioning and should help to elucidate molecular mechanisms that regulate poorly characterized tissues, such as the presumed hydropressure receptors. Expression and functional analyses of the identified genes will clarify the molecular mechanisms governing the complex architecture and networks of the chordate brain.

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References

Acampora, D., Postiglione, M.P., Avantaggiato, V., Di Bonito, M., Vaccarino, F.M., Michaud, J., Simeone, A., 1999. Progressive impairment of developing neuroendocrine cell lineages in the hypothalamus of mice lacking the Orthopedia gene. Genes Dev. 13, 2787–2800.

Adams, B.A., Tello, J.A., Erchegyi, J., Warby, C., Hong, D.J., Akinsanya, K.O., Mackie, G.O., Vale, W., Rivier, J.E., Sherwood, N.M., 2003. Six novel gonadotropin-releasing hormones are encoded as triplets on each of two genes in the protochordate, *Ciona intestinalis*. Endocrinology 144, 1907–1919.

Altman, J., Bayer, S.A., 1978. Development of the diencephalon in the rat. II. Correlation of the embryonic development of the hypothalamus with the time of origin of its neurons. J. Comp. Neurol. 182, 973–994.

Aoyama, M., Kawada, T., Fujie, M., Hotta, K., Sakai, T., Sekiguchi, T., Oka, K., Satoh, N., Satake, H., 2008. A novel biological role of tachykinins as an up-regulation of oocyte growth: identification of an evolutionary origin of tachykinergic functions in the ovary of the ascidian, *Ciona intestinalis*. Endocrinology 149, 4346–4356.

Balment, R.J., Lu, W., Weybourne, E., Warne, J.M., 2006. Arginine vasotocin a key hormone in fish physiology and behaviour: a review with insights from mammalian models. Gen. Comp. Endocrinol. 147, 9–16.

Bosch, T.C., Fujisawa, T., 2001. Polyps, peptides and patterning. Bioessays 23, 420–437.

Campbell, R.K., Satoh, N., Degnan, B.M., 2004. Piecing together evolution of the vertebrate endocrine system. Trends Genet. 20, 359–366.

Chang, C.Y., Liu, Y.X., Zhu, Y.T., Zhu, H.H., 1985. In: Carlick, D.G., Korner, P.I. (Eds.), Frontiers in Physiological Research. : The Reproductive Endocrinology of Amphioxus. Autstralian Academy of Science, Canberra, pp. 79–86.

Caracciolo, A., Gesualdo, I., Branno, M., Aniello, F., Di Lauro, R., Palumbo, A., 1997. Specific cellular localization of tyrosinase mRNA during *Ciona intestinalis* larval development. Dev. Growth Differ. 39, 437–444.

Christiaen, L., Davidosn, B., Kawashima, T., Powell, W., Nolla, H., Vranizan, K., Levine, M., 2008. The transcription/migration interface in heart precursors of *Ciona intestinalis*. Science 320, 1349–1352.

Chomczynski, P., Sacchi, N., 1987. Single-step method of RNA isolation by acid guanidinium thicyanate–phenol–choloroform extraction. Anal. Biocem. 162, 156–159.

Cole, A.G., Meinertzhagen, I.A., 2004. The central nervous system of the ascidian larva: mitotic history of cells forming the neural tube in late embryonic *Ciona intestinalis*. Dev. Biol. 271, 239–262.

Collins 3rd, J.J., Hou, X., Romanova, E.V., Lambrus, B.G., Miller, C.M., Saberi, A., Sweedler, J.V., Newmark, P.A., 2010. Genome-wide analyses reveal a role for peptide hormones in planarian germline development. PLoS Biol. 12, 8, 10, e1000509.

D'Aniello, S., D'Aniello, E., Locascio, A., Memoli, A., Corrado, M., Russo, M.T., Aniello, F., Fucci, L., Brown, E.R., Branno, M., 2006. The ascidian homolog of the vertebrate homeobox gene Rx us essential for ocellus development and function. Differentiation 74, 222–234.

Dehal, P., Satou, Y., et al., 2002. The draft genome of *Ciona intestinalis*: insight into chordate and vertebrate origins. Science 298, 2157–2167.

Deyts, C., Casane, D., Vernier, P., Bourrat, F., Joly, J.-S., 2006. Morphological and gene expression similarities suggest that the ascidian neural gland may be osmoregulatory and homologous to vertebrate per-ventricular organs. Eur. J. Neurosci. 24, 2299–2308.

Dufour, H.D., Chettouh, Z., Deyts, C., de Rosa, R., Goridis, C., Joly, J.S., Brunet, J.F., 2006. Precraniate origin of cranial motoneurons. Proc. Natl Acad. Sci. USA 103, 8727–8732.

Etani, K., Nishikata, T., 2002. Novel G-protein-coupled receptor gene expressed specifically in the entire neural tube of the ascidian *Ciona intestinalis*. Dev. Genes Evol. 212, 447–451.

Flamant, F., Samarut, J., 1998. Involvement of thyroid hormone and its alpha receptor in avian neurulation. Dev. Biol. 197, 1–11.

Goldstein, D.L., 2006. Regulation of the avian kidney by arginine vasotocin. Gen. Comp. Endocrinol. 147, 78–84.

Hamada, M., Wada, S., Kobayashi, K., Satoh, N., 2007. Novel genes involved in *Ciona intestinalis* embryogenesis: characterization of gene knockdown embryos. Dev. Dynm. 236, 1820–1831.

Hartenstein, V., 2006. The neuroendocrine system of invertebrates: a developmental and evolutionary perspective. J. Endocrinol. 190, 555–570.

Harvey, S., Johnson, C.D., Sanders, E.J., 2001. Growth hormone in neural tissues of the chick embryo. J. Endocrinol. 169, 487–498.

Hino, K., Satou, Y., Yagi, K., Satoh, N., 2003. A genomewide survey of developmentally relevant genes in *Ciona intestinalis*. II. Genes for homeobox transcription factors. Dev. Genes Evol. 213, 222–234.

Horie, T., Kusakabe, T., Tsuda, M., 2008. Glutamatergic networks in the *Ciona intestinalis* larva. J. Comp. Neurol. 10, 249–263.

Horie, T., Nakagawa, M., Sasakura, Y., Kusakabe, T.G., 2009. Cell type and function of neurons in the ascidian nervous system. Dev. Growth Differ. 51, 207–220.

Horie, T., Shinki, R., Ogura, Y., Kusakabe, T.G., Satoh, N., Sasakura, Y., 2011. Ependymal cells of chordate larvae are stem-like cells that form the adult nervous system. Nature 469, 525–528.

Hudson, C., Lemaire, P., 2001. Induction of anterior neural fates in the ascidian *Ciona intestinalis*. Mech. Dev. 100, 189–203.

Ikuta, T., Yoshida, N., Satoh, N., Saiga, H., 2004. *Ciona intestinalis* Hox gene cluster: Its dispersed structure and residual colinear expression in development. Proc. Natl Acad. Sci. USA 101, 15118–15123.

Ikuta, T., Saiga, H., 2007. Dynamic change in the expression of developmental genes in the ascidian central nervous system: revisit to the tripartite model and the origin of the midbrain-hindbrain boundary region. Dev. Biol. 312, 631–643.

Imai, K.S., Satoh, N., Satou, Y., 2002. Region specific gene expressions in the central nervous system of the ascidian embryo. Gene Exp. Patt. 2, 319–321.

Imai, K., Hino, K., Yagi, K., Satoh, N., Satou, Y., 2004. Gene expression profiles of transcription factors and signaling molecules in the ascidian embryo: towards a comprehensive understanding of gene networks. Development 131, 4047–4058.

Imai, K., Stolfi, A., Levine, M., Satou, Y., 2009. Gene regulatory networks underlying the compartmentalization of the *Ciona* central nervous system. Development 136, 285–293.

Imai, J.H., Meinertzhagen, I.A., 2007. Neurons of the ascidian larval nervous system in *Ciona intestinalis*: I. Central nervous system. J. Comp. Neurol. 501, 316–334.

Islam, A.F., Moly, P.K., Miyamoto, Y., Kusakabe, T.G., 2010. Distinctive expression patterns of Hedgehog pathway genes in the *Ciona intestinalis* larva: implications for a role of Hedgehog signaling in postembryonic development and chordate evolution. Zoolog. Sci. 27, 84–90.

Jean, D., Bernier, G., Gruss, P., 1999. Six6 (Optx2) is a novel murine Six3-related homeobox gene that demarcates the presumptive pituitary/hypothalamic axis and the ventral optic stalk. Mech. Dev. 84, 31–40.

Jiang, D., Smith, W., 2002. An ascidian engrailed gene. Dev. Genes Evol. 212, 399–402.
Kamesh, N., Aradhyam, G.K., Manoj, N., 2008. The repertoire of G protein-coupled receptors in the sea squirt *Ciona intestinalis*. BMC Evol. Biol. 8, 129.
Kawada, T., Sekiguchi, T., Itoh, Y., Ogasawara, M., Satake, H., 2008. Characterization of a novel vasopressin/Oxytocin superfamily peptide an its receptor from an ascidian, *Ciona intestinalis*. Peptides 29, 1672–1678.
Kawada, T., Sekiguchi, T., Sakai, T., Aoyama, M., Satake, H., 2010. Neuropeptides, hormone peptides, and their receptors in *Ciona* intestinalis: an update. Zoolog. Sci. 27, 134–153.
Kawada, T., Aoyama, M., Okada, I., Sakai, T., Sekiguchi, T., Ogasawara, M., Satake, H., 2009. A novel inhibitory gonadotropin-releasing hormone-related neuropeptide in the ascidian, *Ciona intestinalis*. Peptides 30, 2200–2205.
Kimura, S., Hara, Y., Pineau, T., Fernandez-Salguero, P., Fox, C.H., Ward, J.M., Gonzalez, F.J., 1996. The T/ebp null mouse: thyroid-specific enhancer-binding protein is essential for the organogenesis of the thyroid, lung, ventral forebrain, and pituitary. Genes Dev. 10, 60–69.
Kozmik, Z., Holland, N.D., Kreslova, J., Oliveri, D., Schubert, M., Jonasova, K., Holland, L.Z., Pestarino, M., Benes, V., Candiani, S., 2007. *Pax-Six-Eya-Dach* network during amphioxus development: conservation *in vitro* but context specificity *in vivo*. Dev. Biol. 306, 143–159.
Kusakabe, T., Kusakabe, R., Kawakami, I., Satou, Y., Satoh, N., Tsuda, M., 2001. *Ci-opsin1*, a vertebrate-type opsin gene, expressed in the larval ocellus of the ascidian *Ciona intestinalis*. FEBS Lett. 506, 69–72.
Kusakabe, T., Yoshida, R., Kawakami, I., Kusakabe, R., Mochizuki, Y., Yamada, L., Shin-I, T., Kohara, Y., Satoh, N., Tsuda, M., Satou, Y., 2002. Gene expression profiles in tadpole larvae of *Ciona intestinalis*. Dev. Biol. 242, 188–203.
Kusakabe, T., Mishima, S., Shimada, I., Kitajima, Y., Tsuda, M., 2003. Structure, expression, and cluster organization of genes encoding gonadotropin-releasing hormone receptors found in the neural complex of the ascidian *Ciona intestinalis*. Gene 322, 77–84.
Lang, R., Gundlach, A.L., Kofler, B., 2007. The galanin peptide family: receptor pharmacology, pleiotropic biological actions, and implications in health and disease. Pharmacol. Ther. 1152, 177–207.
López, F.J., Merchenthaler, I., Ching, M., Wisniewski, M.G., Negro-Vilar, A., 1991. Galanin: a hypothalamic–hypophysiotropic hormone modulating reproductive functions. Proc. Natl Acad. Sci. USA 88, 4508–4512.
Manni, L., Lane, N.J., Sorrentino, M., Zaniolo, G., Burighel, P., 1999. Mechanism of neurogenesis during the embryonic development of a tunicate. J. Comp. Neurol. 412, 527–541.
Manni, L., Agnoletto, A., Zaniolo, G., Burighel, P., 2005. Stomodeal and neurohypophysial placodes in *Ciona intestinalis*: insights into the origin of the pituitary gland. J. Exp. Zol. B Mol. Dev. Evo. 304, 324–339.
Mechenthaler, I., 2008. Galanin and the neuroendocrine axes. Cell. Molec. Life Sci. 65, 1826–1835.
Marino, R., Mellilo, D., Di Filloppo, M., Yamada, A., Pinto, M.R., De Santis, R., Brown, E.R., Matassi, G., 2007. Ammonium channel expression is essential for brain development and function in the larva of *Ciona intestinalis*. J. Comp. Neurol. 503, 135–147.
Meinertzhagen, I.A., Okamura, Y., 2001. The larval ascidian nervous system: the chordate brain from its small beginnings. Trends Neurosci. 24, 401–410.
Meinertzhagen, I.A., Lemaire, P., Okamura, Y., 2004. The neurobiology of the ascidian tadpole larva: recent developments in an ancient chordate. Annu. Rev. Neurosci. 27, 453–485.
Merkler, D.J., 1994. C-terminal amidated peptides: production by the in vitro enzymatic amidation of glycine-extended peptides and the importance of the amide to bioactivity. Enzyme Microb. Technol. 16, 450–456.
Mochizuki, Y., Satou, Y., Satoh, N., 2003. Large-scale characterization of genes specific to the larval nervous system in the ascidian *Ciona intestinalis*. Genesis 36, 62–71.
Moret, F., Christiaen, L., Deyts, C., Blin, M., Vernier, P., Joly, J.-S., 2005. Regulatory gene expressions in the ascidian ventral sensory vesicle: evolutionary relationships with the vertebrate hypothalamus. Dev. Biol. 277, 567–579.
Nakagawa, M., Orii, H., Yoshida, N., Jojima, E., Horie, T., Yoshida, R., Haga, T., Tsuda, M., 2002. Ascidian arrestin (Ci-arr), the origin of the visual and nonvisual arrestins of vertebrates. Eur. J. Biochem. 269, 5112–5118.
Nakashima, Y., Kusakabe, T., Kusakabe, R., Terakita, A., Shichida, Y., Tsuda, M., 2003. Origin of the vertebrate visual cycle: genes encoding retinal photoisomerase and two putative visual cycle proteins are expressed in whole brain of a primitive chordate. J. Comp. Neurol. 26, 180–190.
Nicol, D., Meinertzhagen, I.A., 1991. Cell counts and maps in the larval central nervous system of the ascidian *Ciona intestinalis* (L). J. Comp. Neurol. 309, 415–429.
Nishida, H., 1987. Cell lineage analysis in ascidian embryos by intracellular injection of a tracer enzyme: III. Up to the tissue restricted stage. Dev. Biol. 121, 526–541.
Nogueiras, R., et al., 2006. Bsx, a novel hypothalamic factor linking feeding with locomotor activity, is regulated by energy availability. Endocrinology 149, 3009–3015.
Nozaki, M., 2008. The hagfish pituitary gland and its putative adenohypophysial hormones. Zoolog. Sci. 25, 1028–1036.
Ohtaki, T., Kumano, S., Ishibashi, Y., Ogi, K., Matsui, H., Harada, M., Kitada, C., Kurokawa, T., Onda, H., Fujino, M., 1999. Isolation and cDNA cloning of a novel galanin-like peptide (GALP) from porcine hypothalamus. J. Biol. Chem. 274, 37041–37045.
Olson, L.E., Dasen, J.M., Ju, B.G., Tollkuhn, J., Rosenfed, M.G., 2003. Paired-like repression/activation in pituitary development. Recent Prog. Horm. Res. 58, 249–261.
Sanders, E.J., Harvey, S., 2008. Peptide hormones as developmental growth and differentiation factors. Dev. Dyn. 237, 1537–1552.
Sasakura, Y., Makabe, K.W., 2001. A gene encoding a new ONECUT class homeodomain protein in the ascidian *Halocynthia roretzi* functions in the differentiation and speciation of neural cells in ascidian embryogenesis. Mech. Dev. 104, 37–48.
Sasakura, Y., Awazu, S., Chiba, S., Satoh, N., 2003. Germ-line transgenesis of the Tc1/mariner superfamily transposone Minos in *Ciona intestinalis*. Proc. Natl. Acad. Sci. USA 100, 7726–7730.
Satake, H., Kawada, T., Nomoto, K., Minakata, H., 2003. Insight into tachykinin-related peptides, their receptors, and invertebrate tachykinins: a review. Zool. Sci. 20, 533–549.
Satake, H., Ogasawara, M., Kawada, T., Masuda, K., Aoyama, M., Minakata, H., Chiba, T., Metoki, H., Satou, Y., Satoh, N., 2004. Tachykinin and Tachykinin receptor of an ascidian, *Ciona intestinalis:* evolutionary origin of the vertebrate Tachykinin family. J. Biol. Chem. 279, 53798–53805.
Satoh, N., 1994. Developmental biology of ascidians. Cambridge University Press, New York.
Satoh, N., 2003. The ascidian tadpole larva: comparative molecular development and genomics. Nat. Rev. Genet. 4, 285–295.
Satoh, N., Satou, Y., Davidson, B., Levine, M., 2003. *Ciona intestinalis*: an emerging model for whole-genome analyses. Trends Genetic. 19, 376–381.
Satou, Y., Takatori, N., Yamada, L., Mochizuki, Y., Hamaguchi, M., Ishikawa, H., Chiba, S., Imai, K., Kano, S., Murakami, S.D., Nakayama, A., Nishino, A., Sasakura, Y., Satoh, G., Shimotori, T., Shin-i, T., Shoguchi, E., Suzuki, M.M., Takada, N., Utsumi, N., Yoshida, N., Saiga, H., Kohara, Y., Satoh, N., 2001. Gene expression profiles in *Ciona intestinalis* tailbud embryos. Development 128, 2893–2904.
Satou, Y., Imai, K.S., Levine, M., Kohara, Y., Rokhsar, D., Satoh, N., 2003. A genomewide survey of developmentally relevant genes in *Ciona intestinalis*: I. Genes for bHLH transcription factors. Dev. Genes Evol. 213, 213–221.
Satou, Y., Kawashima, T., Shoguchi, E., Nakayama, A., Satoh, N., 2005. An integrate database of the ascidian, *Ciona intestinalis*: Towards functional genomics. Zool. Sci. 22, 837–843.
Satou, Y., et al., 2008. Improved genome assembly and evidence-based global gene model set for the chordate *Ciona intestinalis*: new insight into intron and operon population. Genome Biol. 9, R152.
Schwanzel-Fukuda, M., Pfaff, D.W., 1989. Origin of luteinizing hormone-releasing hormone neurons. Nature 338, 161–164.
Sekiguchi, T., Suzuki, N., Fujiwara, N., Aoyama, M., Kawada, T., Sugase, K., Murata, Y., Sasayama, Y., Ogasawara, M., Satake, H., 2009. Calcitonin in a protochordate, *Ciona intestinalis*—the prototype of the vertebrate calcitonin/aclcitonin gene-related peptide superfamily. FEBS J. 276, 4437–4447.
Sherwood, N.M., Tello, J.A., Roch, G.J., 2006. Neuroendocrinology of protochordates: insights from *Ciona* genomics. Comp. Biochem. Physiol. A. 44, 254–271.
Shimada, M., Nakamura, T., 1973. Time of neuron origin in mouse hypothalamic nuclei. Exp. Neurol. 41, 105–108.
Shimozono, N., Ohta, N., Satoh, N., Hamada, M., 2010. Differential regional expression of genes in the developing brain of *Ciona intestinalis* embryos. Zoolog. Sci. 27, 103–109.
Sheikh, S.P., 1991. Neuropeptide Y and peptide YY: major modulators of gastrointestinal blood flow and function. Am. J. Physiol. 261, G701–G715.
Sower, S.A., Freamat, M., Kavanaugh, S.I., 2009. The origins of the vertebrate hypothalamic-pituitary-gonadal (HPG) and hypothalamic-pituitary-thyroid (HPT) endocrine systems: new insights from lampreys. Gen. Comp. Endocrinol. 161, 20–29.
Steiner, D.F., 1998. The proprotein convertases. Curr. Opin. Chem. Biol. 2, 31–39.
Takamura, K., 1998. Nervous network in larvae of the ascidian *Ciona intestinalis*. Dev. Genes Evol. 208, 1–8.
Takatori, N., Satou, Y., Satoh, N., 2002. Expression of *hedgehog* genes in *Ciona intestinalis* embryos. Mech. Dev. 116, 235–238.
Tassy, O., Dauga, D., Daian, F., Sobral, D., Robin, F., Khoueiry, P., Salgado, D., Fox, V., Caillol, D., Schiappa, R., Laporte, B., Rios, A., Luxardi, G., Kusakabe, T., Joly, J.S., Darras, S., Christiaen, L., Contensin, M., Auger, H., Lamy, C., Hudson, D., Rothbacher, U., Gilchrist, M.J., Makabe, K.W., Hotta, K., Fujiwara, S., Satoh, N., Satou, Y., Lemaire, P., 2010. The ANISEED database: Digital representation, formalization, and elucidation of a chordate developmental program. Genome Res. 20, 1459–1468.
Tata, J.R., 2006. Amphibian metamorphosis as a model for the developmental actions of thyroid hormones. Mol. Cell. Endocrinol. 246, 10–20.
Tatemoto, K., Carlquist, M., Mutt, V., 1982. Neuropeptide Y – a novel brain peptide with structural similarities to peptide YY and pancreatic polypeptide. Nature 296, 659–660.
Tello, J.A., Rivier, J.E., Sherwood, N.M., 2005. Tunicate gonadotropin-releasing hormone (GnRH) peptides selectively activate *Ciona intestinalis* GnRH receptors and green monley type II GnRH receptor. Endocrinology 146, 4061–4073.
Terakado, K., 2001. Induction of Gamete Release by Gonadotropin-Releasing Hormone in a Protochordate, *Ciona intestinalis*. Gen. Comp. Endocrinol. 124, 277–284.
Terakado, K., 2009. Placode formation and generation of gonadotropin-releasing hormone (GnRH) neurons in ascidians. Zoolog Sci. 26, 398–405.
Tessmar-Raible, K., Raible, F., Christodoulou, F., Guy, K., Rembold, M., Hausen, H., Arendt, D., 2007. Conserved sensory-neurosecretory cell types in annelid and fish forebrain: insights into hypothalamus evolution. Cell 29, 1389–1400.
Wada, H., Satoh, N., 2001. Patterning the protochordate neural tube. Curr. Opin. Neurobiol. 11, 16–21.
Wada, H., Saiga, H., Satoh, N., Holland, P.W.H., 1998. Tripartite organization of the ancestral chordate brain and the antiquity of placodes: insights from ascidian Pax-2/5/8, Hox and Otx genes. Development 125, 1113–1122.
Wada, S., Katsuyama, Y., Sato, Y., Itoh, C.S., Saiga, H., 1996. Hroth, an orthodenticle-related homeobox gene of the ascidian, *Halocynthia roretzi*: its expression and putative roles in the axis formation during embryogenesis. Mech. Dev. 60, 59–71.
Wada, S., Tokuoka, M., Shoguchi, E., Kobayashi, K., DiGregorio, A., Spagnuolo, A., Branno, M., Kohara, Y., Rokhsar, D., Levine, M., Saiga, H., Satoh, N., Satou, Y., 2003. A genomewide survey of developmentally relevant genes in *Ciona intestinalis*: II. Genes for homeobox transcription factors. Dev. Genes Evol. 213, 222–234.

Wimalawansa, S.J., 1997. Amylin, calcitonin gene-related peptide, calcitonin, and adrenomedullin: a peptide superfamily. Crit. Rev. Neurobiol. 11, 167–239.
Wray, S., Grant, P., Gainer, H., 1989. Evidence that cells expressing luteinizing hormone releasing hormone mRNA in the mouse are derived from progenitor cells in the olfactory placode. Proc. Natl Acad. Sci. USA 86, 8132–8136.
Yagi, K., Satou, Y., Mazet, F., Shimeld, S.M., Degnan, B., Rokhsar, D., Levine, M., Kohara, Y., Satoh, N., 2003. A genomewide survey of developmentally relevant genes in *Ciona intestinalis*: III. Genes for Fox, ETS, nuclear receptors and NFκB. *Dev*. Genes Evol. 213, 235–244.
Yamada, L., Kobayashi, K., Degnan, B., Satoh, N., Satou, Y., 2003. A genomewide survey of developmentally relevant genes in *Ciona intestinalis*: IV. Genes for HMG transcriptional regulators, bZip and GATA/Gli/Zic/Snail. *Dev*. Genes Evol. 213, 245–253.
Yamada, L., Kobayashi, K., Satou, Y., Satoh, N., 2005. Microarray analysis of localization of maternal transcripts in eggs and early embryos of the ascidian, *Ciona intestinalis*. Dev. Biol. 284, 536–550.
Yoshida, R., Sakurai, D., Horie, T., Kawakami, I., Tsuda, M., Kusakabe, T., 2004. Identification of neuron-specific promoters in *Ciona intestinalis*. Genesis 39, 130–140.
Zega, G., Biggiogero, M., Groppelli, S., Candiani, S., Oliveri, D., Parodi, M., Pestarinom, M., De Bernardi, F., Pennati, R., 2008. Developmental expression of glutamic acid decarboxylase and of gamma-aminobutyric acid type B receptors in the ascidian *Ciona intestinalis*. J. Comp. Neurol. 506, 489–505.